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Th1 and Th2 Cytokines in Mice with Invasive Aspergillosis

ELIO CENCI,[1] STEFANO PERITO,[1] KARL-HEINZ ENSSLE,[2] PAOLO MOSCI,[1] JEAN-PAUL LATGÉ,[3] LUIGINA ROMANI,[1]* AND FRANCESCO BISTONI[1]

Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, 06122 Perugia, Italy[1]; Behringwerke AG, Marburg/Lahn, Germany[2]; and Aspergillus Laboratory, Institut Pasteur, 75015 Paris, France[3]

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With a murine model of invasive aspergillosis we investigated cytokine production by $CD4^+$ T helper cells and the effects of cytokine administration or neutralization on the course and outcome of infection. Patterns of susceptibility and resistance to infection were obtained with different strains of mice injected with different inocula of *Aspergillus fumigatus* conidia. Mice surviving the primary infection also resisted a subsequent lethal infection that was associated with production of gamma interferon by $CD4^+$ T splenocytes. Impaired neutrophil antifungal activity, observed in susceptible mice, was concomitant with a predominant production of interleukin-4 (IL-4) by $CD4^+$ splenocytes. In these mice, exogenous administration of IL-12 failed to induce resistance to infection; in contrast, treatment with soluble IL-4 receptor cured more than 70% of the mice from primary infection and resulted in the onset of acquired resistance to a subsequent lethal infection. These findings indicate that in murine invasive aspergillosis, production of IL-4 by $CD4^+$ T cells may be one major factor discriminating susceptibility and resistance to infection.

Aspergillus fumigatus, the major causative agent of aspergillosis, is a ubiquitous and opportunistic fungus that elicits respiratory infections, such as sinusitis, allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (2, 32). In the immunosuppressed or neutropenic host, invasive pulmonary aspergillosis, characterized by hyphal invasion and destruction of pulmonary tissue, is the most common manifestation of *Aspergillus* infection, although local infections may also occur (2). Increased incidence of pulmonary and invasive aspergillosis has been noted recently for patients with AIDS (6, 13).

Although much attention has been devoted to the study of the enzymes potentially involved in host tissue invasion, no single hydrolase or toxin has been shown to play a role in the pathogenic mechanisms of *A. fumigatus* infection (12, 23, 27, 46). In contrast, numerous studies have shown that the susceptibility to *Aspergillus* infections is dependent on host immunosuppression. Thus, patients at risk for invasive aspergillosis include those undergoing chronic glucocorticoid or immunosuppressive treatment (11, 26) or those with impaired neutrophil or macrophage function, as in chronic granulomatous disease (5), as well as patients with AIDS (6, 13) and neutropenic patients with aplastic anemia (47) or chemotherapy-induced neutropenia (10).

In mice, both innate (29, 45) and acquired immunity (7, 19) contribute to resistance to invasive aspergillosis. It has been shown that neutropenia alone is not sufficient to render mice susceptible to *A. fumigatus* infection, unless the macrophage line of defense is overcome by high challenge doses, activated conidia, or cortisone suppression of macrophage conidiacidal activity (8, 45). However, only a few studies have dealt with acquired immunity to *Aspergillus* infection. It has become clear in recent years that the antigen-specific immune response results in selective or preferential stimulation of $CD4^+$ T helper (Th)-cell subsets. The activation of Th-cell subsets leads to patterns of cytokine secretion and unique T-cell functions. For humans, antigen-specific T cells from patients with allergic bronchopulmonary aspergillosis were characterized as being $CD4^+$ Th2 like in their cytokine synthesis pattern (14). With mice, Kurup et al. demonstrated that the BALB/c strain had high levels of circulating immunoglobulin E (IgE) and eosinophils and produced interleukin-4 (IL-4) and IL-5 in response to particulate *Aspergillus* antigens (16, 17, 24), indicating the occurrence of a Th2 response in experimental allergic aspergillosis. Recently, Nagai et al. have shown that gamma interferon (IFN-γ) and tumor necrosis factor alpha have protective roles in mice with invasive aspergillosis (25). To further define the involvement of the different Th-cell subsets and cytokines in invasive aspergillosis, in the present study we assessed parameters of Th immunity in a murine model of *A. fumigatus* infection and found that the occurrence of protective and nonprotective immune responses correlates with the preferential induction of either one of the two $CD4^+$ Th-cell subsets. Susceptibility to infection was associated with production of IL-4, while IL-4 neutralization rendered susceptible mice capable of healing infection and developing acquired protective immunity.

MATERIALS AND METHODS

Mice. Female inbred BALB/c ($H\text{-}2^d$), DBA/2 ($H\text{-}2^d$), C57BL6 ($H\text{-}2^b$), and hybrid CD2F1 ($H\text{-}2^d/H\text{-}2^d$) mice (Charles River, Calco, Italy), 8 to 10 weeks of age, were used. Animals were kept under standard laboratory conditions.

Microorganism, culture conditions, and infections. The strain of *A. fumigatus* was obtained from a patient with a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of the University of Perugia, Perugia, Italy. The microorganism was grown on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) supplemented with chloramphenicol for 4 days at room temperature. Conidia were harvested by washing the slant culture with 5 ml of 0.025% Tween 20 in normal saline and gently scraping the conidia from the mycelium with a plastic pipette. Cell debris was allowed to settle by gravity, and the suspension was decanted into a 50-ml plastic conical tube. After extensive washing of the conidia with saline, conidia were counted and diluted to the desired concentrations. The viability of the conidia was >95%, as determined by serial dilution and plating the inocula on Sabouraud dextrose agar. For primary infections, mice were injected intravenously (i.v.) via the lateral tail vein with various inocula of *A. fumigatus* conidia in 0.5 ml of saline. For secondary infection, mice were injected i.v. with 10^7 conidia 14 days after primary challenge. Mice suc-

* Corresponding author. Mailing address: Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochetto, 06122 Perugia, Italy. Phone: 39-75-585-3411. Fax: 39-75-585-3413. E-mail: lromani@unipg.it.

cumbing to fungal challenge were routinely necropsied for histopathological confirmation of invasive aspergillosis. For histology, tissues were excised and immediately fixed in formalin. Sections (3 to 4 μm) of paraffin-embedded tissues were examined microscopically for pathologic changes and fungal hyphae after being stained with periodic acid-Schiff. The Gomori methenamine silver staining procedure was always used to confirm the presence of fungal cells.

Chitin assay. For quantification of fungal growth in the organs, the chitin assay was used (7, 18). Lung, brain, and kidney samples were homogenized in 5 ml of 0.9% NaCl, centrifuged, resuspended in 4 ml of 3% sodium lauryl sulfate (Sigma, St. Louis, Mo.), and heated at 100°C for 15 min. After cooling, the pellet was washed once with distilled water, resuspended in 3 ml of 120% (wt/vol) KOH solution, and heated at 130°C for 1 h. After cooling, the alkaline solution was mixed with 8 ml of ice-cold 75% (vol/vol) ethanol and the tubes were shaken until the alkaline solution and ethanol formed a single phase. The tubes were kept at 4°C for 15 min, and 0.3 ml of Celite suspension (the supernatant remaining after 1 g of Celite 545 [Sigma] was mixed with 75% ethanol and left to stand for 2 min) was added. After centrifugation, the supernatant was discarded and the pellet was washed once with 10 ml of ice-cold ethanol (40% [vol/vol]) and twice with cold (4°C) distilled water. To the pellet containing chitosan, 0.5 ml of $NaNO_2$ (5% [wt/vol]) and 0.5 ml of $KHSO_4$ (5% [wt/vol]) were added. All tubes were mixed three times during a 15-min period and then centrifuged. A 12.5% aqueous ammonium sulfamate solution (0.2 ml) was added to 0.6 ml of the supernatant, and the tubes were mixed vigorously for 5 min. Then, 0.2 ml of a 0.5% 3-methyl-2-benzothiazolinone hydrazone aqueous solution was added and the tubes were heated in a boiling water bath for 3 min and cooled. At that time, 0.2 ml of $FeCl_36H_2O$ (0.83% [wt/vol]) was added. The tubes were kept at room temperature for 30 min, and then the optical densities at 650 nm were read with a spectrophotometer. The chitin content of the organs was expressed as micrograms of glucosamine per organ.

In vivo analysis and treatments. For differential blood counts, animals were bled through the tail vein and smears were stained with May-Grünwald Giemsa reagents (Sigma) before analysis. Absolute numbers of polymorphonuclear (PMN) cells in peripheral blood were determined from total and differential white cell counts. Soluble IL-4 receptor (sIL-4R; Boehringer Mannheim) was injected intraperitoneally (i.p.) at doses of 0.1 and 1 μg the day before and 1, 3, and 5 days after challenge (30). Recombinant murine IL-12 (rIL-12; Genetics Institute, Cambridge, Mass.) was given i.p. at the doses of 0.1 and 1 μg per injection twice on the day of challenge and 1 and 2 days later (40). Control mice received the vehicle alone. Endotoxin was removed from all solutions with Detoxigel (Pierce Chemical, Rockford, Ill.).

Killing of hyphae in vitro by PMN cells. The capacity of PMN cells from infected mice to kill hyphae in vitro was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (20, 31). Briefly, PMN cells were obtained from the peritoneal cavities of mice 18 h after injection of 1 ml of aged thioglycolate. Differential cell counts after Diff-Quick (Merck, Darmstadt, Germany) staining of cytospin preparations revealed the presence of 85 to 90% of the neutrophils. Neutrophils (5×10^5) were added to 5×10^4 conidia, which had been cultured at 37°C in 5% CO_2 with 2% fetal calf serum for 16 to 18 h in 96-well flat-bottomed microtiter plates (Costar, Cambridge, Mass.). By that time, more than 95% of the conidia had germinated to hyphae (~150 to ~200 μm in length). After 2 h at 37°C the supernatants were aspirated, effector cells were lysed by addition of sodium deoxycholate (0.5%), and the hyphal viability was determined by MTT staining.

Selection of $CD4^+$ cells and cytokine generation and measurement. Levels of IFN-γ, IL-4, and IL-10 were measured in sera and culture supernatants of activated splenocytes from infected mice (5×10^6 cells were stimulated for 48 h with 10 μg of Concanavalin A [ConA]/ml [Sigma]). In selected experiments, $CD4^+$ lymphocytes were positively selected three times from pools of spleen cells by means of sequential adherence on anti-Ig-coated plates followed by adherence on anti-murine CD4 (monoclonal antibody [MAb] GK1.5), which resulted in a >95% pure population by fluorescence-activated cell sorter analysis (Becton Dickinson & Co., Mountain View, Calif.). Supernatants from mitogen-stimulated lymphocyte cultures were obtained by culturing 5×10^6 $CD4^+$ lymphocytes in the presence of 10 μg of ConA/ml and 5×10^5 accessory macrophages, as described previously (36, 38–41, 43). Sources and characteristics of the anticytokine antibody reagents used in IFN-γ, IL-4, and IL-10 enzyme-linked immunosorbent assays (ELISA) have been previously described in detail (36, 38–41, 43). Briefly, sera and cell supernatants were tested for their concentrations of IFN-γ with rat anti-murine IFN-γ MAb R4-6A2 as the primary antibody and biotinylated MAb AN-18.17.24 as the secondary antibody. For IL-4 and IL-10 measurements, two-site ELISA involved the use of MAb 11B11, in combination with biotinylated monoclonal BVD6-24G2, and MAb SXC-2, in combination with biotinylated SXC-1 (PharMingen, San Diego, Calif.), respectively. All cytokine titers were calculated by reference to standard curves constructed with known amounts of recombinant IFN-γ, IL-4 (Genzyme, Boston, Mass.), and IL-10 (PharMingen).

RNA preparation and RT-PCR. $CD4^+$ lymphocytes, unfractionated splenocytes, or plastic-adherent macrophages (>95% pure on esterase staining) were obtained from spleen cells pooled from three to four animals, and 2×10^7 cells were subjected to RNA extraction by the guanidium thiocyanate-phenol-chloroform procedure as described previously (4). Briefly, 3 μg of total RNA was incubated with 0.5 μg of oligo(dT) (Pharmacia, Uppsala, Sweden) for 3 min at 65°C and chilled on ice for 5 min. Each sample was then incubated for 2 h at 42°C after addition of 20 U of RNase inhibitors (Boehringer Mannheim Italia, Milan, Italy), 1.5 mM deoxynucleoside triphosphates, 25 U of avian myeloblastosis virus reverse transcriptase (RT; Boehringer Mannheim), and RT buffer (50 mM Tris-HCl [pH 8.3], 8 mM $MgCl_2$, 30 mM KCl, 10 mM dithiothreitol, final concentrations) in a final volume of 20 μl. The cDNA was diluted to a total volume of 500 μl with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and frozen at −20°C until use. Amplification of synthesized cDNA from each sample was carried out as described previously (44). Briefly, 5 μl of cDNA was added to a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.0 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, 1 μM each primer, and 2.5 U of AmpliTaq polymerase (Perkin-Elmer Corp., Hayward, Calif.). Each 100-μl sample was overlaid with 75 μl of mineral oil (Sigma) and incubated in a DNA Thermal Cycler 480 (Perkin-Elmer Corp.) for a total of 30 cycles for each cytokine. For amplification of β-actin- and IFN-γ-specific primers, each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. For IL-4, each cycle consisted of 1 min at 94°C, 1 min at 67°C, and 1 min at 72°C; for IL-10, each cycle consisted of 10 s at 91°C, 25 s at 59°C, and 25 s at 72°C; for IL-12p40, each cycle consisted of 1 min at 94°C, 1 min at 67°C, and 1 min at 72°C; and for IL-12p35, each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The primers and positive controls were as described elsewhere (36, 38–41). The β-actin primers were used as a control for both the reverse transcription and the PCR itself and also for comparing the amounts of products from samples obtained with the same primer. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR-assisted mRNA amplification was repeated at least twice for at least two separately prepared cDNA samples for each experiment. The reported data are representative of at least three different experiments. Under these conditions, control samples taken from uninfected animals showed no background cytokine mRNA levels, so the magnitude of the immune response to infection could be easily demonstrated.

Statistical analysis. In all in vivo experiments, six to eight animals per group were used. Mortality data were analyzed by the Mantel-Haenzel test for survivorship data. Fisher's exact test was applied to the proportions of mice surviving and dying in the control and treated groups. Student's *t* test was used to determine statistical significance between *A. fumigatus* killing activities and the chitin contents of the experimental groups. *P* value of <0.05 was considered statistically significant. The data reported in this study are pooled from three to five experiments.

RESULTS

Course of invasive aspergillosis in different strains of mice. As the establishment of primary pulmonary aspergillosis in mice was found to be dependent on the inoculum of the fungus (8), we evaluated the course of invasive aspergillosis in different strains of mice upon injection of different inocula of fungal cells. Mice were injected i.v. with 10^6 or 10^7 cells, assessed for resistance to infection and reinfection, and monitored for mortality and fungal growth in the organs. The results showed that all strains of mice succumbed to infection with the highest inoculum of the fungus in 3 to 4 days (Table 1). All strains of mice also succumbed to infection with 5×10^6 cells (data not shown). In contrast, C57BL/6, BALB/c, and CD2F1, but not DBA/2, mice survived the infection with 10^6 fungal cells. On assaying resistance to subsequent infection with 10^7 conidia, it was found that surviving mice also resisted a second challenge given 14 days later. Resistance was still present at 21 days after primary infection (data not shown), as already shown by others (7). To correlate these findings with fungal growth in the kidneys and lungs, we used an assay for the measurement of the cell wall chitin (18) to quantitate *A. fumigatus* in infected tissues. The results show that at 2 days after infection, extensive fungal growth was evident in the kidneys and lungs of the different strains of mice and, particularly, DBA/2 mice (Table 1). Fungal cells could also be detected in the brains of infected animals (data not shown). On assaying the persistence of fungal cells in organs of CD2F1 mice, it was found that fungal cells were present at 7, 14, and 21 days after infection and still present 40 days later (Fig. 1). Renal aspergillosis was confirmed by histopathology of the kidneys. Figure 2A shows the presence of severe invasive aspergillosis involving a medulla and characterized by necrotized inflammation extending to the renal cortex of the kidneys of CD2F1 mice infected with a

TABLE 1. Courses of invasive aspergillosis in different strains of mice

Mouse strain	No. of conidia in inoculum for primary infection[a]	Chitin content[b]		Resistance to infection			
				Primary		Secondary[c]	
		Kidneys	Lungs	MST[d]	D/T[e]	MST	D/T
C57BL6	10^7	ND	ND	4	20/20		
C57BL6	10^6	26.4 ± 2.8	18.2 ± 2.8	>60	0/16	>60	0/4
DBA/2	10^7	ND	ND	3	20/20		
DBA/2	10^6	39.3 ± 5.3[f]	27.8 ± 4.0[g]	6	20/20		
BALB/c	10^7	ND	ND	3	24/24		
BALB/c	10^6	16.1 ± 3.3	13.0 ± 2.2	>60	0/24	>60	0/4
CD2F1	10^7	ND	ND	3.5	24/24		
CD2F1	10^6	17.4 ± 1.6	18.5 ± 2.7	>60	2/24	>60	0/4

[a] Mice were injected i.v. with different numbers of *A. fumigatus* conidia.
[b] Chitin contents (in micrograms of glucosamine per organ) were determined 2 days after infection. ND, not determined.
[c] In one experiment, half of the mice surviving primary infection were challenged 14 days later with *A. fumigatus* conidia (10^7, i.v.).
[d] MST, median survival time (days).
[e] D/T, number of dead mice over total number of animals injected.
[f] $P < 0.01$ (DBA/2 mice versus all strains) according to Student's t test.
[g] $P < 0.01$ (DBA/2 mice versus BALB/c mice); $P < 0.05$ (DBA/2 mice versus C57BL6 and CD2F1 mice).

lethal inoculum of *Aspergillus*. Numerous hyphae and inflammatory PMN cells were present. In contrast, only a few hyphae were present in the medullas of mice infected with a sublethal inoculum of the fungus. Moreover, lesions present in the cortices of these mice exhibited a pattern of healing, with a few abscesses containing predominantly mononuclear inflammatory cells (Fig. 2B).

$CD4^+$ Th-cell-subset activation in mice with invasive aspergillosis. To correlate susceptibility and resistance to both primary and secondary infections with parameters of Th immunity, CD2F1 mice were injected with either a lethal or a sublethal inoculum of *A. fumigatus* and assessed for cytokine production in vitro. Splenocytes, obtained from mice at 2 and 7 days after infection, were mitogen stimulated in vitro before assessment of IFN-γ, IL-4, and IL-10 contents in the culture supernatants. The results (Fig. 3) show that production of IFN-γ was lower in mice lethally injected than in mice surviving infection, particularly at 2 days after infection. In contrast, IL-4 production decreased in mice surviving infection and was significantly lower than that observed in susceptible mice, particularly at 7 days after infection. Moreover, although no differences in IL-10 production were observed in resistant and susceptible mice at 2 days postinfection, there was a significantly decreased production in mice surviving infection. IFN-γ, but not IL-4, IL-10, or IL-12p70, was detected in the sera of infected mice (data not shown). To correlate these findings with the presence of $CD4^+$ Th1 and Th2 cells in vivo, purified populations of $CD4^+$ splenocytes from infected mice were assessed for IFN-γ, IL-4, and IL-10 gene expression by RT-PCR; IL-12p40 expression in splenic macrophages was also assessed. The results (Fig. 4) show that messages for IFN-γ and IL-10 were always present, being detectable at all inocula every day. In contrast, the IL-4 message weakened late in infection in mice injected with the sublethal inoculum of the fungus, at the time when the IL-12p40 message could still be detected.

Antifungal activities of PMN cells in invasive aspergillosis. In a murine model of disseminated candidiasis, a direct correlation was found between the fungal load and the activation of IL-4 gene expression, such that impairment of neutrophil antifungal effector functions resulted in an increased fungal burden in the tissues and in the activation of $CD4^+$ Th2 cells (42). As neutrophils are the first line of defense against *Aspergillus* hyphae (45), we investigated neutrophil antifungal effector functions in mice showing patterns of resistance or susceptibility to invasive aspergillosis. In both infections, the numbers of circulating neutrophils, determined by tail bleeds at different days of infection, increased over time with a peak at 7 days, when the majority of mice infected with a lethal inoculum of the fungus would die. In contrast, in mice surviving infection the number of neutrophils declined after 7 days but was still high at 2 weeks after infection (Fig. 5). To evaluate the antifungal potential of PMN cells, their ability to kill hyphae in vitro was assessed in the early days of infection. The killing capacity of PMN cells was highly increased in mice resistant to infection but not in those succumbing to infection (Table 2). In the latter mice, a marked decrease in killing capacity was observed 3 days after infection.

Effect of cytokine administration or neutralization on the course of invasive aspergillosis. Because cytokine administration or neutralization profoundly affects the course of experimental fungal infections (15, 21), we studied the effects of IL-4 inhibition and rIL-12 administration in mice with invasive aspergillosis. With an established treatment schedule (30, 40), CD2F1 mice were injected i.v. with a lethal inoculum of *Aspergillus* conidia and treated with different doses of sIL-4R or rIL-12. The results (Table 3) show that treatment with sIL-4R, but not rIL-12, resulted in the cure of more than 70% of the infected mice. Cure was associated with decreased fungal burdens in the kidneys and lungs, as opposed to the increased fungal loads observed in IL-12-treated mice. Surviving mice also showed increased resistance to a subsequent, otherwise lethal, challenge with the fungus, with a median survival time of 48 days upon rechallenge, as compared to 4 days for naive control mice. These results indicate that inhibition of IL-4 could benefit mice with invasive aspergillosis, thus suggesting that production of IL-4 may exacerbate the infection.

DISCUSSION

The results of this study show that in a murine model of invasive aspergillosis, the ability of $CD4^+$ Th cells to release

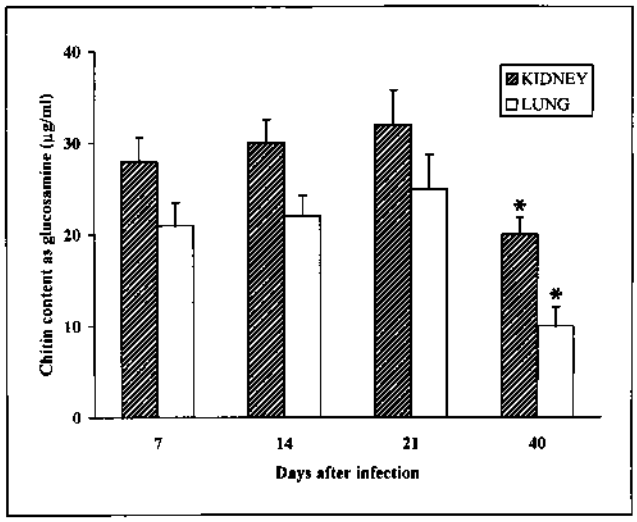

FIG. 1. Chitin contents in the kidneys and lungs of CD2F1 mice inoculated i.v. with 10^6 viable conidia of *A. fumigatus* on various days after infection. T bars represent standard deviations. *, $P < 0.05$ (40 days versus 7 days; kidney samples), $P < 0.01$ (40 days versus 14 and 21 days; kidney samples) and $P < 0.01$ (40 days versus all days; lung samples).

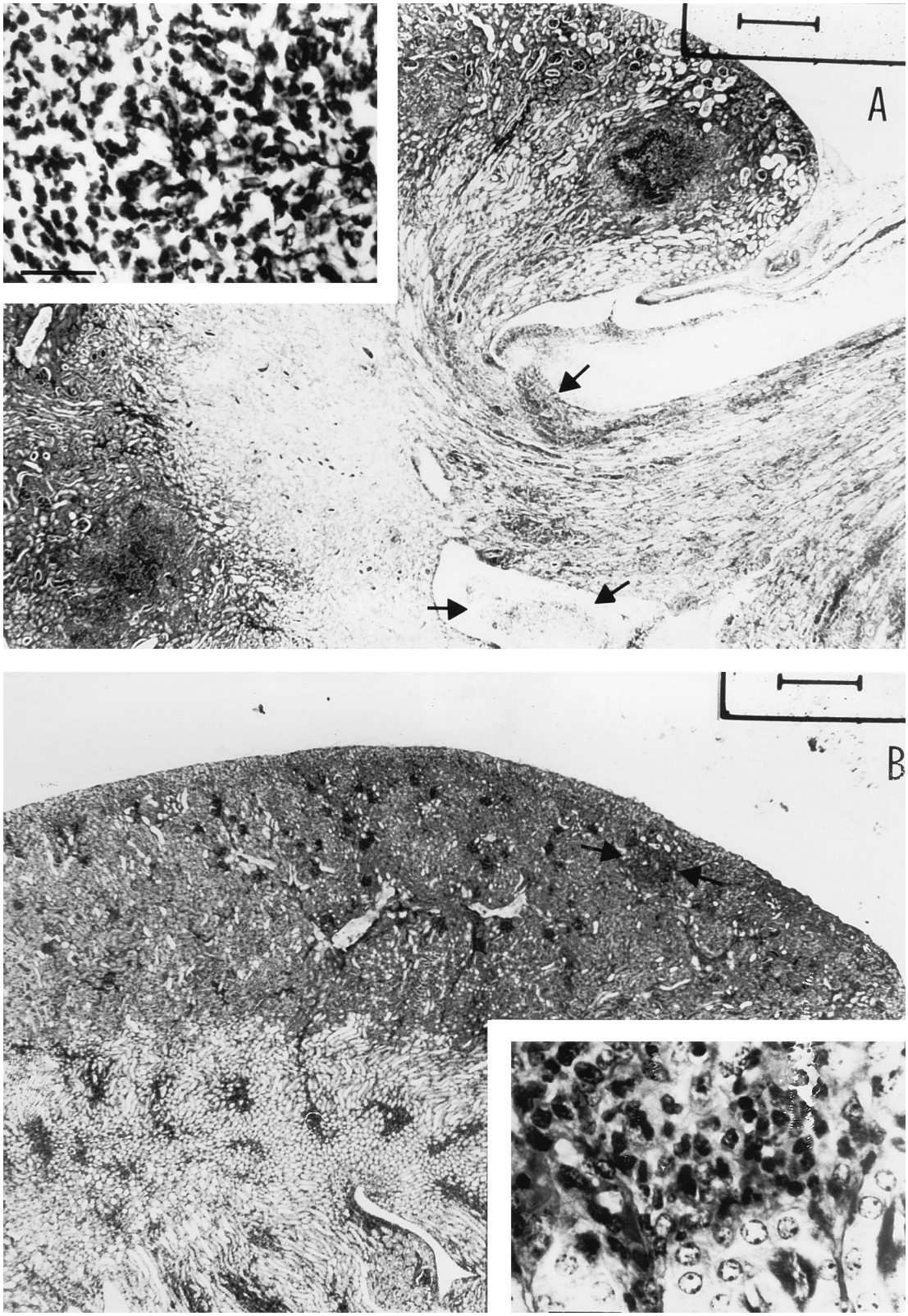

FIG. 2. Histologic analysis of kidneys of mice with invasive aspergillosis. Periodic acid-Schiff-stained sections were prepared from the kidneys (largest transverse diameter) of mice at 1 week after infection with a lethal (5×10^6) (A) or nonlethal (10^6) (B) inoculum of *A. fumigatus*. (A) Severe invasive aspergillosis (bar = 400 μm) involving the medulla and characterized by the presence of numerous hyphae and extensive acute inflammatory exudate extending to the renal cortex (magnified in the inset; bar = 50 μm). (B) Healing inflammatory lesions with no evidence of fungal growth in the cortex (bar = 400 μm). The inset depicts a magnified area showing a lymphomononuclear infiltrate (bar = 50 μm).

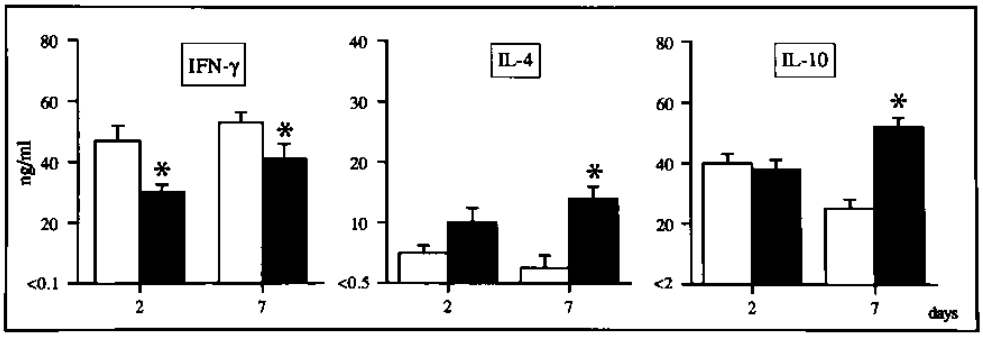

FIG. 3. Production of IFN-γ, IL-4, and IL-10 by splenocytes from CD2F1 mice injected with a sublethal (10^6, □) or a lethal (5×10^6, ■) inoculum of *A. fumigatus*. At 2 and 7 days after infection, 5×10^6 splenocytes (pooled from 6 mice) were stimulated for 48 h with ConA (10 μg/ml), before assessment of cytokine contents in culture supernatants by ELISA. Cytokine levels in culture supernatants of mitogen-stimulated splenocytes from uninfected mice were 8 ± 0.4 ng/ml for IFN-γ, 2 ± 0.4 ng/ml for IL-4, and 10 ± 1 ng/ml for IL-10. Cytokine levels in supernatants of unstimulated infected splenocytes were below the detection limits of the assays (indicated as the amount preceeded by a < on the *y* axis). *, $P < 0.05$ (IFN-γ) and $P < 0.01$ (IL-4 and IL-10).

cytokines is differentially regulated in mice that are resistant and mice that are susceptible to infection. Production of IL-4 occurred predominantly in mice injected with a lethal inoculum of the fungus. Conversely, in mice injected with a sublethal inoculum of virulent fungal cells, a predominant production of IFN-γ was observed.

It has already been reported that sublethal i.v. infection results in a protective effect on subsequent i.v. challenge with *A. fumigatus* conidia (7, 19, 48) and that the acquired immunity is mediated by macrophages (7). Here we show that development of protective acquired immunity is associated with the presence of $CD4^+$ cells producing IFN-γ and macrophages producing IL-12. However, administration of rIL-12 failed to increase resistance in nonhealing mice, which confirms the inability of IL-12 to exert protective effects in mouse models of invasive fungal infection (35, 40). In contrast, IFN-γ, which is known to potentiate the antifungal activity of human phagocytic cells (1, 31, 34), has recently been found to have a protective effect in mice with invasive aspergillosis (25). In that study, treatment with IFN-γ rescued the infected animals from death, and this rescue was associated with early increased expression of both IFN-γ- and IL-12p40-specific messages in the spleen. Conversely, IFN-γ neutralization resulted in an increased pathology and a concomitant increased expression of the IL-10 message, thus suggesting that a differential expansion of the Th1 and Th2 $CD4^+$ cell subsets had occurred in mice that are resistant and mice that are susceptible to invasive aspergillosis.

While in agreement with these findings, the results of our study further demonstrate the occurrence of $CD4^+$ Th2-cell activation by showing that $CD4^+$ cells from susceptible mice produce IL-4, in addition to IL-10. The production of IL-4 strictly correlated with the course and outcome of infection, as its depletion early in infection resulted in decreased fungal loads in the organs and the cure of mice from infection. Surviving mice developed resistance to a subsequent lethal infection, thus indicating the onset of protective acquired immunity. These results are reminiscent of those obtained with a murine model of disseminated candidiasis, in which resistance and susceptibility to infection were causally related to the dissimilar expansion of functionally distinct $CD4^+$ Th-cell subsets (21, 30, 35–43). Th1 cells confer protection in genetically resistant mice and in susceptible mice treated with IL-4 (21, 30, 39) or IL-10 (21, 43) antagonists. In contrast, Th2 responses are associated with disease progression and the onset of nonprotective responses, as observed in mice treated with IFN-γ (36)- or IL-12 (40, 42)-neutralizing antibody. Whether the $CD4^+$ Th-cell subsets operate similarly in the two types of infection remains to be elucidated. In murine candidiasis, production of IL-4 by $CD4^+$ Th2 cells correlates with the presence of high levels of circulating IgE (38). In contrast, in our model of invasive aspergillosis, antigen-specific, circulating IgE could be detected only in BALB/c mice, regardless of the type of infection (data not shown). This finding indicates that, in addition to the route of administration and the physical form of the *Aspergillus* antigen (16, 17), the genetic background of the host is an important factor in the expression of high levels of IgE in murine models of aspergillosis.

One major issue in microbial pathogenesis is to understand how the interaction of microorganisms with the innate immune system might shape the subsequent cognate immune response orchestrated by T cells. In this regard, neutrophils and macrophages represent the first two lines of defense against *A. fumigatus* infection (45). Pulmonary alveolar macrophages ingest and kill inhaled conidia, while PMN leukocytes and monocytes

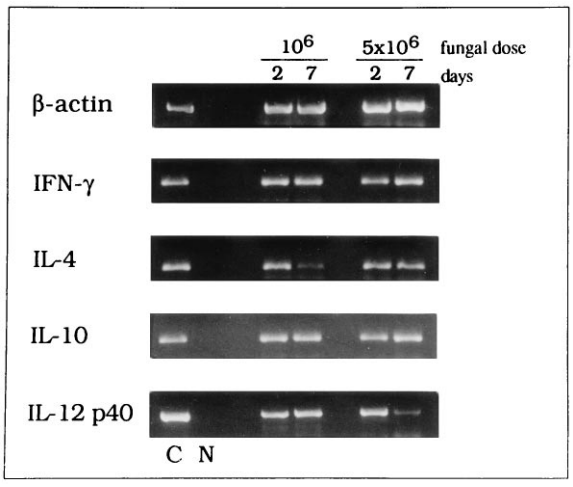

FIG. 4. Analysis of cytokine gene expression in mice with invasive aspergillosis. CD2F1 mice were injected i.v. with a lethal (5×10^6) or sublethal (10^6) inoculum of *A. fumigatus* conidia. At 2 and 7 days after infection, purified $CD4^+$ T cells and macrophages from spleen cells (pooled from six mice) were assessed for cytokine gene expression (IFN-γ, IL-4, and IL-10 in $CD4^+$ T cells; IL-12p40 in macrophages) by RT-PCR. C, β-actin or cytokine-specific controls; N, no DNA was added to the amplification mixture during PCR.

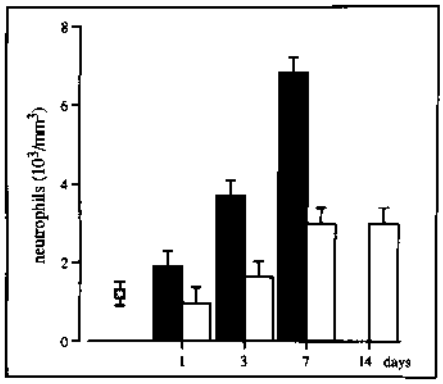

FIG. 5. Levels of circulating neutrophils in mice with *A. fumigatus* infection. Absolute numbers of peripheral blood neutrophils were determined by tail bleeds in mice at different days after i.v. injection of 5×10^6 (■) or 10^6 (□) *A. fumigatus* conidia. ▨, number of neutrophils in uninfected control mice. Values represent the means ± standard errors of data from 20 mice tested individually in four separate experiments.

are fungicidal to the hyphal form. The importance of innate resistance in host defense against this infection is suggested by the association of invasive aspergillosis with severe defects of neutrophil and macrophage antifungal effector functions (5). Here we show that the ability of neutrophils to kill hyphal elements was upregulated in mice surviving the infection and severely depressed in mice succumbing to it. In the latter, a concomitant decrease in nitric oxide (NO) production was also observed (data not shown), although the precise role of NO in the killing of *Aspergillus* conidia has yet to be defined (22). Interestingly, the quantitative changes in neutrophil antifungal activity were most evident at 3 days after infection, a finding which could indicate the appearance of factors with positive and negative effects on neutrophil antifungal effector functions. In this regard, it is worth mentioning that cytokines may have opposite effects on antifungal effector functions of phagocytic cells (3, 9, 15). Therefore, it is likely that neutrophils, through regulation of the fungal burden in the tissues, actively participate in the generation of the subsequent adaptive Th response, which in turn modulates their antifungal activities. If this proves to be true for humans, then the increasing recognition of invasive aspergillosis in patients with late- or end-stage human immunodeficiency virus (HIV) disease (6, 13) may likely reflect the dysregulation of Th cytokine production,

TABLE 2. Ability of neutrophils from mice with invasive aspergillosis to kill hyphal cells in vitro

A. fumigatus infection[a]		Mean % of hyphae killed ± SD[b]
No. of conidia in inoculum	Day	
None		8.7 ± 2.1
10^6	1	28.8 ± 3.4[c]
10^6	3	36.4 ± 4.2[c]
5×10^6	1	16.5 ± 2.3[d]
5×10^6	3	1.8 ± 0.7[c]

[a] CD2F1 mice were injected i.v. with *A. fumigatus* conidia and assessed for neutrophil antifungal effector functions 1 day or 3 days later.
[b] *A. fumigatus* conidia (5×10^4) were incubated for 16 to 18 h in the presence of 2% fetal calf serum before addition of 5×10^5 neutrophils and assessment of fungal cell viability by the MTT assay.
[c] $P < 0.01$ (infected versus control mice) according to Student's *t* test.
[d] $P < 0.05$ (infected versus control mice).

TABLE 3. Effect of treatments with sIL-4R or rIL-12 on the course of invasive aspergillosis

Group	Treatment[a]		*A. fumigatus* infection		Chitin content[b]	
	Type	Dose (μg)	MST[c]	D/T[d]	Kidneys	Lungs
1	None		9	20/20	27.2 ± 2.2	23.8 ± 1.7
2	sIL-4R	0.1	11.5	18/18	ND[e]	ND
3	sIL-4R	1	>60[f, g]	5/18[h]	12.7 ± 0.6[i]	10.1 ± 0.8[i]
4	rIL-12	0.1	6.5	18/18	48.4 ± 3.4[j]	39.7 ± 3.3[j]
5	rIL-12	1	10	18/18	ND	ND

[a] CD2F1 mice were injected i.v. with *A. fumigatus* conidia (5×10^6) and treated i.p. with sIL-4R (the day before and 1, 3, and 5 days after infection) or rIL-12 (twice on the day of challenge and 1 and 2 days later).
[b] The chitin contents (in micrograms of glucosamine per organ) were determined 1 week after infection.
[c] MST, median survival time (days).
[d] D/T, number of dead mice over total number of animals injected.
[e] ND, not determined.
[f] A parallel group of mice was injected i.v. with 10^7 *A. fumigatus* conidia 14 days after the primary challenge. Upon rechallenge, the median survival time of sIL-4R-treated mice was 48 days as opposed to 4 days for control, uninfected mice.
[g] $P < 0.01$ (group 3 versus group 1) according to the Mantel-Haenzel test.
[h] $P < 0.01$ (group 3 versus group 1) according to Fisher's exact test.
[i] $P < 0.01$ (group 3 versus group 1) according to Student's *t* test.
[j] $P < 0.01$ (group 4 versus group 1) according to Student's *t* test.

which negatively affects antifungal effector function of neutrophils. In this regard, it is worth mentioning that both qualitative and quantitative changes in neutrophils are observed in advanced HIV infection (28) and that neutrophil activity against *A. fumigatus* is severely impaired in HIV-infected children (33).

Because invasive aspergillosis occurs mainly in immunosuppressed or neutropenic patients (2, 32), it will be important to assess the interplay between the innate and specific immune systems in a mouse model of invasive aspergillosis that may mimic human aspergillosis. In any case, the results obtained with our model of infection, while indicating what the appropriate antifungal response should be, show that a better understanding of the reciprocal regulation between the different Th subsets and cytokine production may be useful in the proper management of invasive aspergillosis.

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